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NITRATE AND NITRITE VOLATILIZATION BY MICOORGANISM IN LABORATORY EXPERIMENTS

Jean-Marc Bollag

Pennsylvania State University

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Microbial nitrate and nitrite volatilization was considered as a means to eliminate nitrogen from soil and water in order to inhibit the accumulation of nitrogenous substances as pollutants or health hazardous compounds. Therefore it was attempted to compare nitrate reducing microorganisms in their reactions to different environmental conditions in laboratory experiments. Changing oxygen concentration, pH, temperature, nitrate or nitrite concentration affected differently the denitrification process of various isolated microorganisms. Unfavorable growth conditions led to the accumulation of nitrite if nitrate served as substrate. It was found that certain soil fungi are also capable of volatilizing nitrogen as nitrous oxide.

Biological and chemical factors were evaluated during nitrite transformation in autoclaved and non-autoclaved soil by determination of the evolvement of nitrogenous gases. During chemical nitrite volatilization, which occurred essentially at a low pH, the major gases evolved were nitric oxide and nitrogen dioxide, but if biological activity was predominant in a neutral and alkaline environment, nitrous oxide and molecular nitrogen were formed. The validity of laboratory observations in relation to field studies in the domain of denitrification is discussed and evaluated. (CPA)

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NITRATE AND NITRITE VOLATILIZATION BY MICROORGANISMS IN LABORATORY EXPERIMENTS

By

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ABSTRACT

Microbial nitrate and nitrite volatilization was considered as a means to eliminate nitrogen from soil and water in order to inhibit the accumulation of nitrogenous substances as pollutants or health hazardous compounds. Therefore it was attempted to compare nitrate-reducing microorganisms in their reactions to different environmental conditions in laboratory experiments.

Changing oxygen concentration, pH, temperature, nitrate or nitrite concentration affected differently the denitrification process of various isolated microorganisms. Unfavorable growth conditions led to the accumulation of nitrite if nitrate served as substrate.

It was found that certain soil fungi are also capable of volatilizing nitrogen as nitrous oxide.

Biological and chemical factors were evaluated during nitrite transformation in autoclaved and non-autoclaved soil by determination of the evolvement of nitrogenous gases. During chemical nitrite volatilization, which occurred essentially at a low pH, the major gases evolved were nitric oxide and nitrogen dioxide, but if biological activity was predominant in a neutral and alkaline environment, nitrous oxide and molecular nitrogen were formed.

The validity of laboratory observations in relation to field studies in the domain of denitrification is discussed and evaluated.

This report was submitted in fulfillment of Project Number R-800997 (16080 EIT - formerly; WP-01536 - formerly), Contract 68-X-0100 under the sponsorship of the Office of Research and Monitoring, Environmental Protection Agency. The experiments were performed in the Laboratory of Soil Microbiology, Department of Agronomy, The Pennsylvania State University, University Park, Pennsylvania 16802.

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SECTION I

CONCLUSIONS

1. Although microbial denitrification appears to be a potential solution to the problem of nitrogen elimination from soil and water (lakes, rivers, etc.), the available knowledge is still fragmentary and inadequate in order to manage efficiently the denitrification process in the various ecosystems. The reported data in this investigation present a contribution to the problem of optimizing denitrification, but considerable more research is needed in order to be able to use the basic research findings under the numerous different natural conditions.

2. There are many indications that unfavorable growth conditions may have a greater effect on the nitrite-reducing enzyme system than on the enzyme which transforms nitrate. Consequently, a temporary or remaining nitrite accumulation can occur when nitrate is reduced in a non-optimal environment.

3. The optimum pH for nitrate reduction and growth of bacteria was found to be in the neutral area. However, the metabolic activity on nitrite has a different pH range for the investigated microbes. Consequently, the interactions which occur in soil by changing pH are complex.

4. Soil fungi can also participate in the volatilization of nitrogen. It could be shown that fungi from the genus Fusarium can reduce nitrite with the simultaneous release of nitrous oxide. Although only approximately 10% of the supplied nitrite substrate was detected in gaseous form, fungi may be of importance in influencing the nitrogen content of the soil since they can account for the largest portion of the total microbial protoplasm in certain soils.

5. It is possible to conclude from our experiments with soil samples that nitrite is converted to gaseous products under aerobic conditions by chemical reactions, whereas at a neutral and alkaline pH the conversion is caused mainly by biological activity. During chemical nitrite volatilization the major gases are nitric oxide and nitrogen dioxide, but if biological activity is predominant, nitrous oxide and molecular nitrogen are formed.

6. Various nitrogen-containing compounds interfere with methane-producing microorganisms. Nitrate caused the strongest inhibition of methane formation followed with decreasing efficiency by nitrite, nitric oxide and nitrous oxide. This observation could provide a means to control methane formation if this process causes an ecological problem.

7. Most laboratory incubation studies leave doubt as to whether the data obtained apply to field conditions. Artificial conditions of the laboratory are, however, requisite if the factors determining the paths of a biological reaction are to be critically evaluated. The study comparing denitrifying microorganisms in liquid media and in soil demonstrates that the physiological reactions of the bacteria are similar in the two media, and consequently basic studies performed in the laboratory may provide valuable indicative results for further evaluation under field conditions.

SECTION II

RECOMMENDATIONS

Additional research on the nitrogen transformation resulting from microbial activities in soil, streams and lakes should be undertaken. Considerable more basic knowledge on denitrifying microorganisms is needed if one is interested to monitor their activity under the various environmental conditions, in order to use the denitrification process for nitrogen removal.

Research should be performed under field conditions as well as in well-controlled laboratory studies. The investigation in the laboratory is still an important prerogative since the conditions in a specific ecosystem like soil are so complex that it is extremely difficult to receive conclusive results concerning a specific activity of microorganisms.

Different approaches in research should be used in order to find effective means for optimizing or stimulating conditions for extensive denitrification:

Nutritional factors which may be selective for the growth and activity of denitrifiers should receive special consideration. Certain organic compounds which serve better as energy sources and more significantly satisfy the nutritional demands of the denitrifying microflora should be added at the appropriate time to stimulate the nitrogen removal process.

It may also be assumed that certain growth factors or other specific substances are required and can be identified by their selective action on the denitrifying group among the microbes.

Compounds which interfere in the nitrogen cycle, i.e., which inhibit nitrogen fixation or nitrification, are also of special interest. It is often assumed that nitrifiers and denitrifiers keep the nitrogen level in soil at a certain equilibrium. An interference in an ecosystem may result in the dominance of one group and consequently alter the physiological balance.

The use of denitrification for removal of nitrogen can also be applied on a small scale. Therefore it would be valuable to develop devices in which the denitrification process can be used in homes and farms for removal of nitrate and nitrite from drinking water.

SECTION III

INTRODUCTION

The accumulation of nitrogen in soil and water causes one of the major problems in pollution of the environment. In soil systems with high concentrations of nitrate-nitrogen, the nitrate-nitrogen can leach into the groundwater where it may present a health hazard. Drinking water with nitrate-nitrogen levels above 8 to 9 ppm causes methemoglobinemia or cyanosis in infants (Report of the 'Environmental Pollution Panel of the White House', 1965). For water given to livestock, a nitrate concentration above 5 ppm nitrate-nitrogen is regarded as unsafe, and high concentrations may result in methemoglobinemia, loss of milk production, vitamin A deficiency, thyroid disturbances, and reproductive difficulties and abortions.

The use of a land disposal system for sewage effluents appears to be a promising method for renovation of waste water, but the removal of nitrogen presents one of the limiting factors for continuous and increasing applications of waste.

Microbial denitrification appears to be a potential solution to the problem of nitrogen elimination. This process, which has been used by modifying a conventional biological sewage treatment system, may have a considerable importance in soil as well as in an aqueous ecosystem. In order to exploit the denitrification reaction for these specific aims it is necessary to understand more clearly the physiological mechanisms which lead to this reaction. The literature on denitrification is voluminous. However, there exist many conflicting reports, and we are still ignorant about essential steps during denitrification, especially its dependence on ecological factors.

It must be remembered that two alternatives for the reduction of nitrites and nitrates by bacteria exist: 1. Assimilative Reduction;

formation of ammonia from NO_3^- or NO_2^-, which is then transferred into the anabolic cell-metabolism. This reductive process merely accumulates the nitrogen in the soil and leaves it available for re-oxidation.
2. Respiratory Reduction, i.e., denitrification in which nitrates or nitrites replace oxygen as final electron acceptors in respiration. The end product of this respiration may be molecular nitrogen or the oxides of nitrogen. The term denitrification has sometimes been used in a much broader sense, but will be used in this report according to the above definition which was approved by the Soil Science Society of America (Proceedings 26, 307).

Microbial reduction of nitrates and nitrites is brought about by a number of species of facultatively anaerobic bacteria. Most of them use oxygen preferentially as a hydrogen acceptor but may also use nitrates and nitrites as substitutes. The end products of this respiration are—as outlined above—nitrogenous gases which are lost from the soil by diffusion into the atmosphere.

It is this mechanism which provokes concern in agricultural practice since the loss of nitrogen in gaseous form was regarded as a loss of fertilizer. Most of the applied research in agriculture has therefore been directed toward eliminating denitrification.

It was the major purpose of this project to find methods to stimulate the microbial denitrification process. This would provide a biological method for nitrate removal in gaseous form when an aqueous or a soil ecosystem is overloaded with nitrogen-rich material from waste disposal. In recent years it has also been recognized that nitrogen-loss in soil involves a combination of biological as well as chemical reactions. Although it was our intention to study especially the biological mechanism, certain chemical processes were also evaluated.

Field investigations of the denitrification process have met with many difficulties. The conditions in a soil ecosystem are so complex that

it is extremely difficult to receive conclusive results concerning a specific microbial process. Especially in the nitrogen cycle it is difficult to know or to distinguish under field conditions if, for example, the denitrification process is partially hidden by nitrifying organisms or if a non-biological factor contributes to additional release of nitrogenous gases. Therefore, it appeared to us that in order to study denitrification and the major influencing factors, it is necessary to have well-controlled conditions and also pure microbial cultures for obtaining conclusive results. The drawback is obvious since the conditions designed in the laboratory have to be quite different from those existing in the microenvironment within the soil. Nevertheless, it appeared that results obtained from pure microbial cultures in liquid solutions or in a soil environment are comparable, and consequently may provide indicative results for further evaluation under field conditions. The artificial conditions of laboratory experiments are requisite if the many ecological factors determining the paths of a biological reaction are to be critically studied.

SECTION IV

METHODS USED FOR THE DETERMINATION OF THE DENITRIFYING ACTIVITY

Nitrate determination

Nitrate was initially measured by the brucine method (American Public Health Association, 1965), but later this determination was abandoned, since the use of the nitrate-electrode proved to be a much less time-consuming procedure and simultaneously the sensitivity and accuracy was quite comparable. A high nitrite concentration interfered especially with the brucine determination. When this happened, the amount of nitrate was determined and interpolated after nitrite determination according to established standard curves. It was very rare that nitrite accumulated in the investigated samples to such an extent that interference constituted a problem.

Nitrite determination

Nitrite was always measured in the aqueous phase by the naphthylamine-sulfanilic acid procedure (American Public Health Association, 1965).

Measurements of nitrogenous and other gases

Gas samples were withdrawn from the flasks with a gas-tight syringe (Hamilton Corp., Whittier, Cal.) and injected into a gas chromatograph (Varian Aerograph 1820) using two parallel columns (3 mm o.d.) simultaneously at 50°C: Porapak Q (600 cm; 50-80 mesh) and Molecular sieve 5A (450 cm; 45-60 mesh). The detectors were dual thermal conductivity cells at a temperature of 200°C. The inlet temperature was 70°C. Helium was used as a carrier gas at a flow rate of 40 ml per minute under 2.95 kg/cm^2 pressure. The filament current was 200 mA for the Porapak Q column and 150 mA for the Molecular sieve 5A column. The quantity of gases was determined by the use of an integrator (Digital Integrator, Model 477, Varian Aerograph) and calibrated with known amounts of the various gases. During incubation of the different

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samples, gases were usually formed which caused a positive pressure in a flask. The increase in gas pressure was measured with a manometric device, and the amount of the formed and detected gases was calculated accordingly. The column of Porapak Q clearly indicated peaks of carbon dioxide (CO_2), nitrous oxide (N_2O), and nitric oxide (NO), whereas the other column of Molecular Sieve 5A separated oxygen and nitrogen (see example in Figure 1).

In certain experiments nitrogen dioxide (NO_2) and nitric oxide (NO) were determined by absorption of these gases in 1 N NaOH-solution containing 125 mMol of $KMnO_4$ per liter. Depending on the expected formation of NO_2 or NO, one or two vials containing 4 ml of the $KMnO_4$ solution were placed in the incubation flask. For further analysis this solution was decolorized according to the procedure of Anderson (1965) and subsequently neutralized and the amounts of nitrite and nitrate were determined.

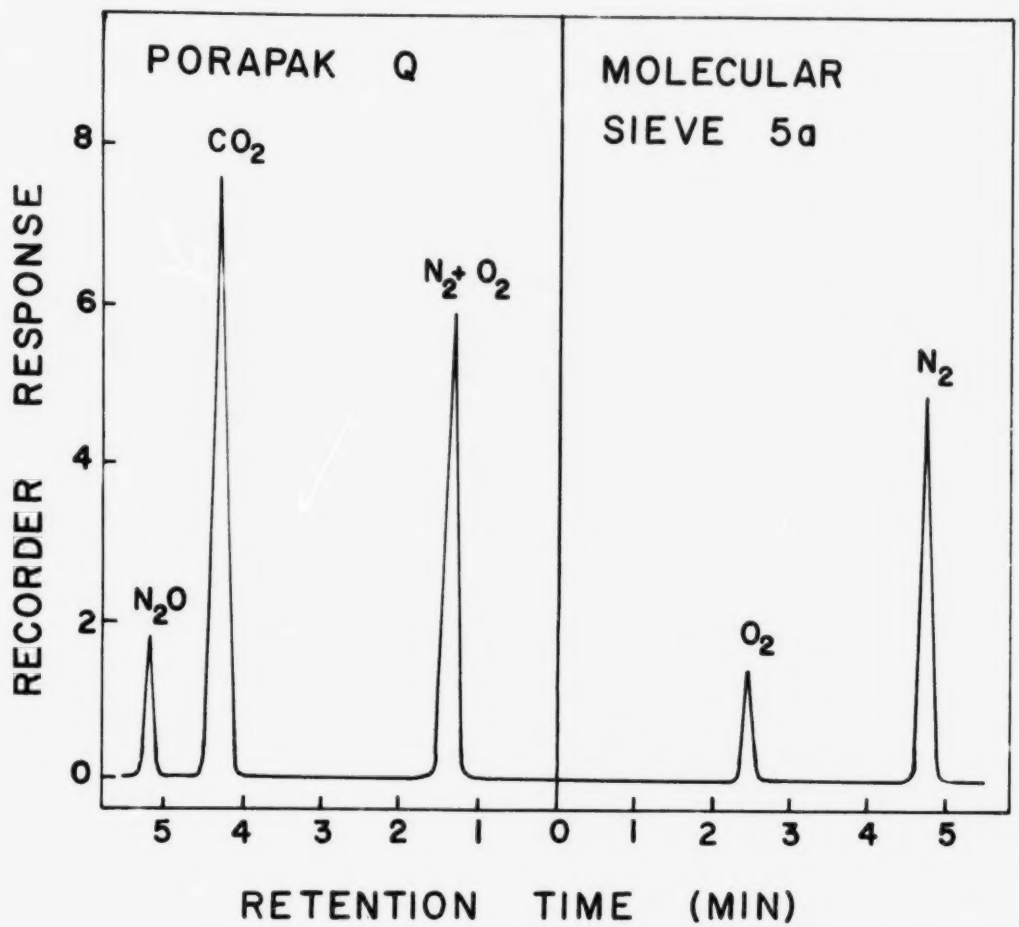

Figure 1: Tracing of a chromatogram revealing the appearance of gases liberated after 3 days incubation of a growth culture of F. oxysporum in 'semi-anaerobic' conditions.

SECTION V

ISOLATION OF DENITRIFYING BACTERIA AND SOME OF THEIR PHYSIOLOGICAL CHARACTERISTICS

Though there exists a considerable literature on denitrification, many reports on the physiological and biochemical characteristics of denitrifying microbes appear contradictory. Many studies have been performed with a single bacterial strain or atypical denitrifier (e.g., E. coli) and consequently general conclusions have been misleading. Therefore, it seemed desirable to isolate various denitrifiers from soil and to compare their physiological activity.

A large number of nitrate-reducing bacteria strains were enriched from soil (Hublersburg silt loam soil) by using anaerobic conditions in a culture solution (Giltay's medium) containing 1.0 g KNO_3, 1.0 g l-asparagine, 8.5 g Na-citrate, 1.0 g KH_2PO_4, 1.0 g $MgSO_4 \cdot 7H_2O$, 0.2 g $CaCl_2 \cdot 6H_2O$, and 0.05 g $FeCl_3 \cdot 6H_2O$ per liter of distilled water which was adjusted to a final pH of 7.2 with NaOH. After several transfers in the liquid medium, the microbes were isolated using the dilution technique and plating on agar. After incubation for several days at 30°C, single colonies were selected and purified by further plating. The resulting pure cultures were transferred at 2-month intervals to fresh slants of nitrate agar. After growth at 28°C, these slants were maintained at 3-5°C and were used for preparation of the liquid cultures.

The experiments under anaerobic conditions were carried out in 250 ml suction flasks fitted with a rubber stopper containing a glass tube that extended into the culture medium. On top of the glass tube and at the suction outlet rubber tubing was attached and sealed airtight with a pinchcock clamp. All flasks contained 90 ml of Giltay's medium and a test tube containing 4 ml of 8N NaOH for absorbing carbon dioxide. In order to provide anaerobic conditions, argon or helium was

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forced through the glass tube for 5 to 10 minutes and allowed to escape through the suction outlet. Then the flask was immediately sealed. The flushing of the incubation flasks was performed at the beginning of an experiment and daily thereafter when samples were withdrawn. All cultures were incubated at 30°C.

Cultures grown in air were obtained by inoculating a 250 ml Erlenmeyer flask containing 60 ml of medium on a gyrotory shaker (200 to 250 oscillations/minute) at 30°C.

Each test was replicated several times; average values are reported. Culture samples were withdrawn daily and observed for growth and gas production. Gas appearance was regarded as an indication of denitrification, but the only certain proof of denitrification in these studies was the actual measurable loss of nitrogen as indicated by total nitrogen determination (Kjeldahl method, Bremner and Shaw, 1958).

After the isolation of approximately 60 nitrate reducing microorganisms from soil, the denitrifying characteristics were established in preliminary experiments. Only four isolates were selected for further investigation since they varied considerably in their nitrate reducing features. Since the microbes were not identified, they will be labelled in this report as Isolate A, D, G, and H. Some morphological and physiological characteristics of these bacteria are summarized in Table 1.

When the selected isolates were cultivated under anaerobic conditions, all nitrate disappeared from the culture medium within 48 to 72 hours' incubation at 30°C. Nitrite was usually not detected in the medium of Isolates A and D. However, nitrite invariably accumulated in the growth medium of Isolates G and H. Nitrite disappeared only with the bacterial strain G after further incubation (Figure 2).

TABLE 1: MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF THE INVESTIGATED NITRATE REDUCING BACTERIA.

Bacterium	Cell Morphology	Motility	Gram reaction	Acid formation from			Gelatin liquefaction	Litmus milk	Pigment	Requirement for oxygen	Nitrate reduction
				Glucose	Sucrose	Lactose					
Isolate A	rod	non-motile	negative	+	+	-	-	alkaline	-	facultative	+
Isolate D	rod	non-motile	negative	+	+	+	-	alkaline	-	facultative	+
Isolate G	rod	motile	negative	-	-	-	-	alkaline	orange (on potato)	facultative	+ temporary nitrite formation
Isolate H	rod	motile	negative	+	-	-	+	alkaline	dark orange (on nitrate agar)	facultative	+ nitrite formation

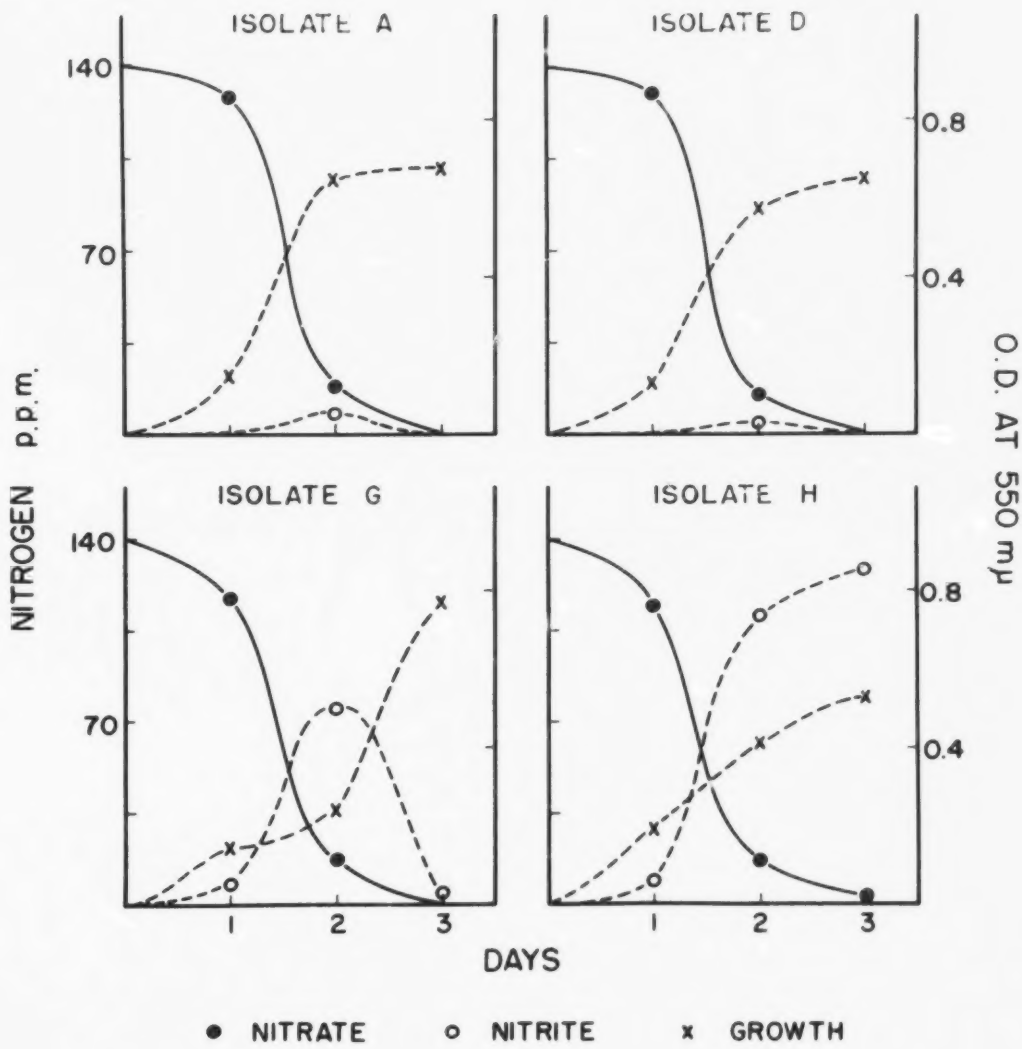

Figure 2: Nitrate reducing characteristics of the isolated micro-organisms during growth under anaerobic conditions.

In order to determine if the disappearance of nitrate and nitrite and the observed development of gases by the isolates could be used as indicators for the denitrifying activity, the total nitrogen was determined in each growth culture under aerobic and anaerobic conditions.

It is evident from Table 2 that a considerable loss of nitrogen occurred with Isolates A and D when comparing the cultures which were exposed to oxygen with those cultured anaerobically. Isolate G showed indications that some nitrogen may be lost even under aerobic conditions.

During anaerobic growth it was observed, as in previous reports, that there was an "alkaline drift" from the original pH of 7.2. The final pH after 3 days' incubation was measured as 8.4, 7.9, 7.6, and 8.1, respectively, for the Isolates A, D, G, and H. Isolate H showed a slight shift to the acid side during the early stages of growth.

TABLE 2. PERCENTAGE OF TOTAL NITROGEN (KJELDAHL DETERMINATION) IN CULTURE MEDIUM AFTER 3 DAYS' GROWTH UNDER AEROBIC AND ANAEROBIC CONDITIONS.

	CULTURE CONDITIONS	
	Aerobiosis	Anaerobiosis
	%N	%N
Isolate A	4.76	2.04
Isolate D	4.91	1.89
Isolate G	4.10	3.04
Isolate H	5.11	2.93
Control medium (uninoculated)	5.01	4.91

SECTION VI

COMPARATIVE DENITRIFICATION OF SELECTED MICROORGANISMS IN CULTURE MEDIA AND IN AUTOCLAVED SOIL

It was also attempted to compare the denitrifying characteristics of the isolated bacteria and of some laboratory cultures in the liquid artificial growth medium and in a sterile soil system in order to clarify if the mere change from a prepared liquid to the solid and complex structure of a soil causes an essential change of denitrification activity by the tested microbes.

Pseudomonas aeruginosa, Serratia marcescens, and Bacillus subtilis which were also used for this study were obtained from the culture collection of the Department of Microbiology at The Pennsylvania State University. The liquid culture solution was 'Giltay's medium' (described in the previous section) and the soil used in these experiments was a Hagerstown silt loam soil with a neutral pH; the indigenous nitrate-nitrogen concentration was 75 µg/g of soil; organic-carbon, 1.80%; and the sand-, silt- and clay-contents were 8.5%, 63.4%, and 28.1%, respectively. Twenty grams of soil were placed into each incubation flask and 10 ml of distilled water containing 5 mg nitrate-nitrogen were added. Sterilization of the soil was accomplished by autoclaving the samples three times for 30 minutes over a 2- to 3-day period with at least 12 hours' interval. To inoculate the soil, 1.0 ml of the selected stock culture grown in Giltay's medium was added to each sample.

Incubation of anaerobic samples was observed in 125 ml bottles which were sealed with a one-hole rubber stopper containing a septum. Anaerobic conditions were attained by flushing the bottles with helium until all air was removed.

In order to incubate liquid media under anaerobic conditions, 50 ml of Giltay's medium were added to each bottle and inoculated with one loop of a bacterial culture.

For aerobic conditions 125 ml Erlenmeyer flasks were used which were stoppered with foam plugs. Soil specimens were placed in a vacuum incubation chamber and a slight negative pressure insured a continuous exchange of air—also within the flasks. Aerobic liquid samples were kept on a rotary incubation shaker revolving at 200 oscillations per minute. All samples—aerobic and anaerobic—were incubated at 30°C for 3 days. The experiments were repeated two or three times and several replicates were evaluated for each specific treatment.

The comparison of the denitrifying characteristics of the selected microorganisms in a liquid growth solution as well as in soil is depicted in Figure 3. Nitrate and nitrite determination as well as the measurement of the evolved gases during incubation served as indicators of the microbial activity. After incubation for 3 days it was apparent that the disappearance of nitrate was slower in the soil than in the liquid Giltay's medium, but the essential features of nitrate transformation by the microbes were not affected under the two tested conditions. Isolates A and D produced a considerable amount of nitrous oxide in the culture medium as well as in soil, but the intermediary formation of nitrite could not be observed. On the other hand, Serratia marcescens and Isolate H showed similar characteristics concerning the marked formation of nitrite in Giltay's medium and the same observation was present but not so apparent under soil conditions. The smaller accumulation of nitrite in soil could be correlated with the slow transformation of nitrate and a lower nitrite concentration could facilitate its subsequent volatilization to nitrogen gas.

The growth of Pseudomonas aeruginosa was very rapid in the culture solution as indicated by increasing turbidity. At the same time it

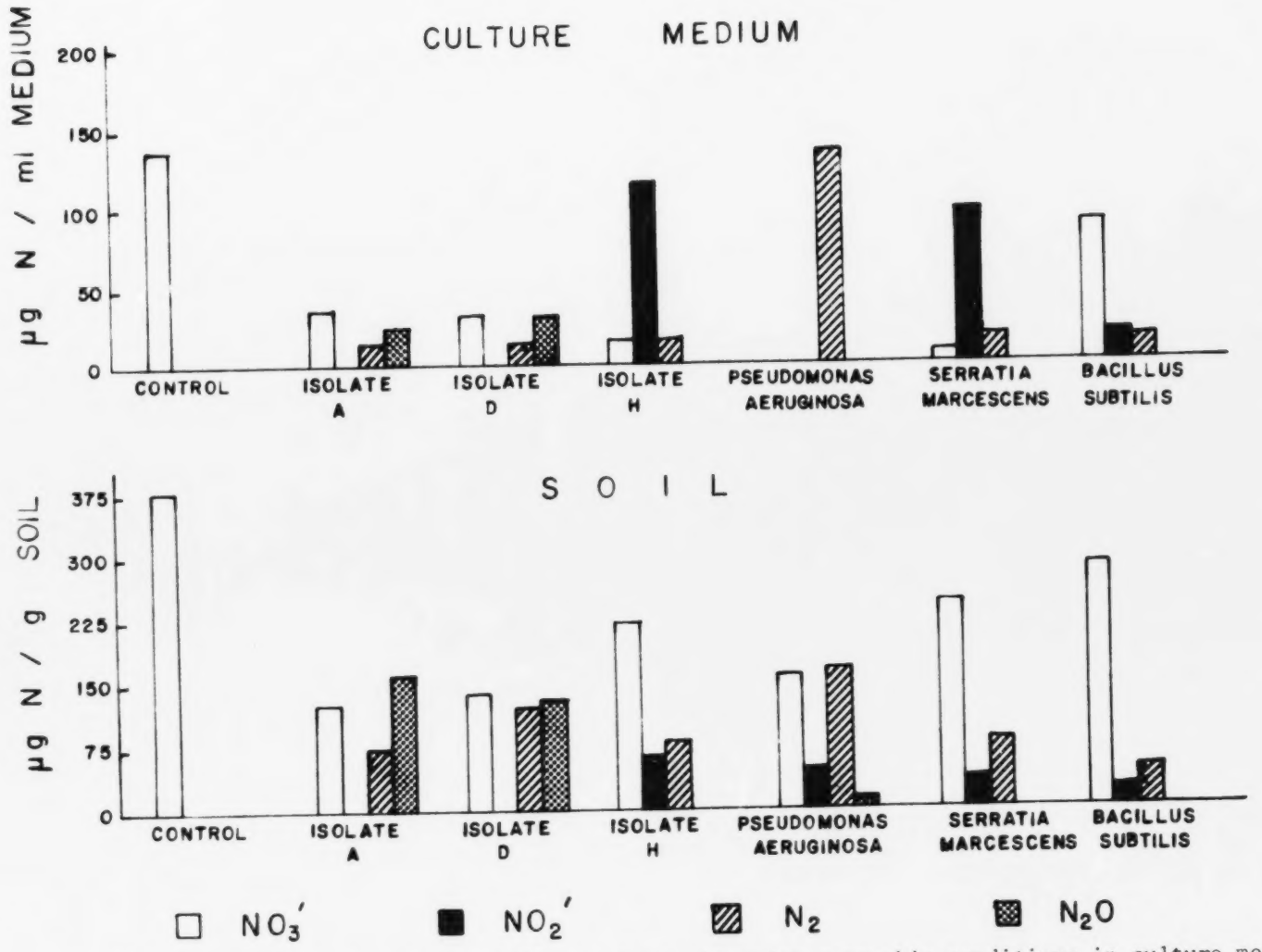

Figure 3: Denitrifying activity of different bacteria under anaerobic conditions in culture media and in soil.

could be found that practically all nitrate which disappeared was recovered as nitrogen gas. However, if the Pseudomonas species was cultivated in soil, it used the nitrate more slowly and, although N_2 was the predominant gas, in this case it was also possible to detect nitrite and some nitrous oxide. Bacillus subtilis was also included in these experiments although this bacterium is not considered to be a denitrifier. It was found that both in soil and in the culture some nitrate was transformed and it could be recovered as nitrite and nitrogen gas.

Simultaneously with the release of the nitrogenous gases the formation of CO_2 was observed and the results of one representative experiment are shown in Table 3. It is of interest to note that Isolate H and Serratia marcescens which accumulate in the culture solution a considerable amount of nitrite show a strong production of CO_2 if compared with the other active bacteria, but a weaker nitrate transforming ability in soil is complemented with a relatively smaller formation of CO_2. This fact is especially noteworthy if it is compared with the bacteria which do not accumulate nitrite: their production of CO_2 is by far more extensive in soil than in the culture medium although less nitrate disappeared. For example, Isolate A produced 10.2 µg of CO_2 per ml culture solution and 69.3 µg of CO_2 per g soil.

A small amount of CO_2 formation which was found in the sterile control of the soil samples appears to be of nonbiological origins, e.g., CO_2 may be produced by decarboxylation of organic compounds or decay of free carbonates.

TABLE 3: CO_2 FORMATION UNDER ANAEROBIC CONDITIONS IN GILTAY'S MEDIUM AND IN SOIL.

	CO_2 - Formation in							
	Giltay's medium (µg/ml)				Soil (µg/g)			
	Sample 1	Sample 2	Sample 3	Average	Sample 1	Sample 2	Sample 3	Average
Control	0	0	0	0	8.7	7.0	7.3	7.7
Isolate A	9.0	10.4	11.3	10.2	137.6	137.0	137.6	137.4
Isolate D	11.0	10.8	18.6	13.4	123.2	125.6	125.5	124.8
Isolate H	29.7	32.9		31.3	71.9	75.5	76.8	74.7
Pseudomonas aeruginosa	16.9	17.7	16.0	16.9	115.7	138.8	114.9	123.1
Serratia marcescens	52.8	52.0	57.9	54.2	69.8	68.7		69.3
Bacillus subtilis	4.4	8.2	8.2	6.9	58.8	61.1	51.4	57.5

SECTION VII

ENVIRONMENTAL FACTORS IN THE DENITRIFYING PROCESS

If one is interested in influencing denitrification in nature by stimulation or inhibition, it is primarily necessary to know the responses of the predominating denitrifiers to the various environmental conditions. Though there exists a considerable literature on these topics, many observations are contradictory, e.g., the question how the oxygen concentration is related to denitrification or what is the most suitable pH for denitrifiers still evokes controversial answers; therefore, it appeared desirable to investigate the influence of various environmental factors on the selected microorganisms in this study.

Denitrification at Different Temperatures

All metabolic processes can be characterized by their temperature dependency. Therefore, the response of Isolates A, D, G, and H to temperatures at 10°, 22°, 30° and 37°C was observed under anaerobic conditions. Since growth was poor at 10°C and little denitrification occurred, only the data for the higher temperatures are reported (Table 4). Best growth and nitrate disappearance were established for all isolates at 30°C which was also the optimal temperature for growth under aerobic conditions. Isolate A did not show any growth at 37°C under either aerobic or anaerobic conditions. An interesting point is that temporary accumulations of nitrite occurred with strain D at 22°C and 37°C after 3 days. This indicates that the nitrate reducing enzyme system of this isolate is more temperature dependent than nitrate reductase. In all cultures there was a clear correlation between the rate of growth and nitrate reduction under the various temperatures.

Effect of pH on Denitrification

It is generally assumed that denitrification is favored in a neutral to alkaline ecosystem and that denitrifying populations in otherwise

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TABLE 4: EFFECT OF TEMPERATURE ON DENITRIFICATION BY ANAEROBICALLY GROWN ISOLATES.

Temperature	Incubation period	Growth, optical density at 550 mμ	Nitrate concentration	Nitrite concentration
°C	days		ppm N	ppm N
		Isolate A		
22	1	0.00	110	0.0
	2	0.02	110	0.0
	3	0.22	96	3.4
	7	0.54	0	0.0
30	1	0.00	110	0.0
	2	0.34	33	0.0
	3	0.46	6	1.2
	7	0.47	0	0.0
37	1	0.00	110	0.0
	2	0.00	110	0.0
	3	0.00	110	0.0
	7	0.00	110	0.0
		Isolate D		
22	1	0.00	110	0.0
	2	0.05	110	0.0
	3	0.21	36	9.3
	7	0.23	0	0.0
30	1	0.04	110	0.0
	2	0.27	12	0.0
	3	0.28	0	0.0
	7	0.32	0	0.0
37	1	0.02	110	0.0
	2	0.36	16	0.0
	3	0.39	0	6.4
	7	0.41	0	0.0
		Isolate G		
22	1	0.00	110	0.0
	2	0.04	102	7.5
	3	0.24	66	28.0
	7	0.85	0	0.0
30	1	0.00	110	0.0
	2	0.21	24	41.0
	3	0.57	0	16.5
	7	0.82	0	0.0
37	1	0.00	110	0.0
	2	0.00	110	0.0
	3	0.04	82	16.3
	7	0.09	70	7.2
		Isolate H		
22	1	0.17	96	2.5
	2	0.43	40	39.0
	3	0.66	21	65.0
	7	0.67	8	76.0
30	1	0.40	43	48.0
	2	0.57	31	51.0
	3	0.74	25	58.0
	7	1.05	17	71.0
37	1	0.00	110	0.0
	2	0.15	102	5.2
	3	0.19	96	8.9
	7	0.22	92	10.2

optimal environmental conditions fail to release gaseous nitrogen at high hydrogen ion concentration (Nommik, 1956). It is not surprising that relatively little denitrifying activity was found at pH 5 and below, since the metabolic activity of most microorganisms is reduced under acidic conditions. However, many contradictory statements exist in the literature on the influence of pH on nitrate volatilization.

Broadbent (1951) reported that denitrification is favored below pH 7, whereas other investigators (Jansson and Clark, 1952; Bremner and Shaw, 1958b; Valera and Alexander, 1961) concluded that nitrogen loss was considerably suppressed under acidic conditions. In another report (Khan and Moore, 1968) it is concluded that no correlation between pH and denitrification parameters could be found.

As in other instances, a general statement on the effect of pH on denitrification can be misleading, since the various microbial strains investigated here showed different responses in their denitrifying behavior at a changing hydrogen ion concentration.

In Figure 4, the data demonstrate the influence of pH on nitrate reduction with the four investigated isolates after 2 days of growth under anaerobic conditions. In no instance was there significant growth or nitrate disappearance below pH 6.0. Best growth occurred at a neutral pH and nitrate disappearance was closely related to this phenomenon, but the accumulation of nitrite varied in relation to the pH with the different organisms. Isolates A and D produced nitrite at pH 8, but not at pH 7. Isolate G formed the largest amount of nitrite at pH 6, although optimal growth and nitrate reduction occurred at pH 7.

Influence of Different Nitrate and Nitrite Concentrations

Information is scarce on the response of the denitrifying population to increasing concentrations of nitrate. This constitutes an important, practical problem in agriculture and waste disposal. Wijler and

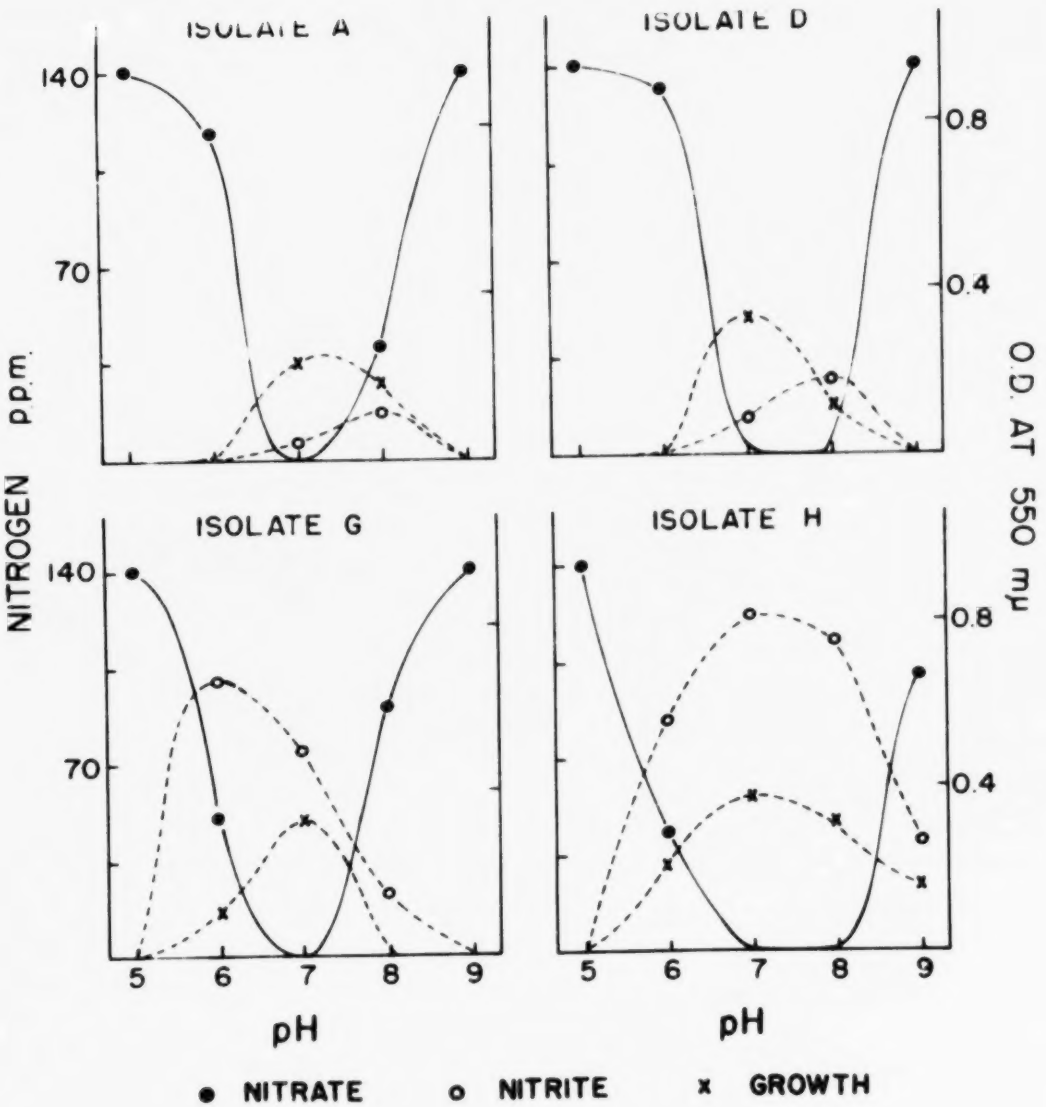

Figure 4: Effects of pH on growth and denitrification (samples taken from 2-day-old anaerobic cultures).

Delwiche (1954) as well as Nommik (1956) concluded from soil experiments that the initial nitrate concentration does not influence the rate of gas release. Nitrite is toxic to various microorganisms, yet it is not known how much can be produced when nitrate is reduced, and to what extent its accumulation may inhibit the denitrifying process.

The four isolates were tested under anaerobic conditions for their ability to use nitrate as well as nitrite at very low to toxic concentrations during 3 days of growth. It appears from the results in Figure 5 that, if the growth medium contains 0.5% nitrate, only Isolate G is able to transform all applied nitrate within 3 days. Isolates A and D use only a small amount, a part of which can be recovered as nitrite. The appearance of considerably more nitrite with higher nitrate concentrations seems to be a general phenomenon. There was no visible inhibitory effect of 2.0% nitrate on the growth of all isolates tested, but relatively little of the added nitrate disappeared.

From Figure 5 it is obvious that the optimal and acceptable concentration for growth is considerably higher with nitrate than with nitrite. One-tenth of 1 percent nitrate proved to be optimal for denitrification by these four soil isolates. The same concentration of nitrite was inhibitory for Isolates A and D; 0.2% nitrite suppressed practically all growth.

Whereas relatively low concentrations of nitrite exerted an inhibitory effect on growth and denitrification, there was no indication that increasing amounts of nitrate suppress the development of microorganisms. However, if at higher concentrations unused nitrate remained in the growth medium, an accumulation of nitrite occurred. This phenomenon requires more attention since it could constitute an important factor under certain ecological conditions.

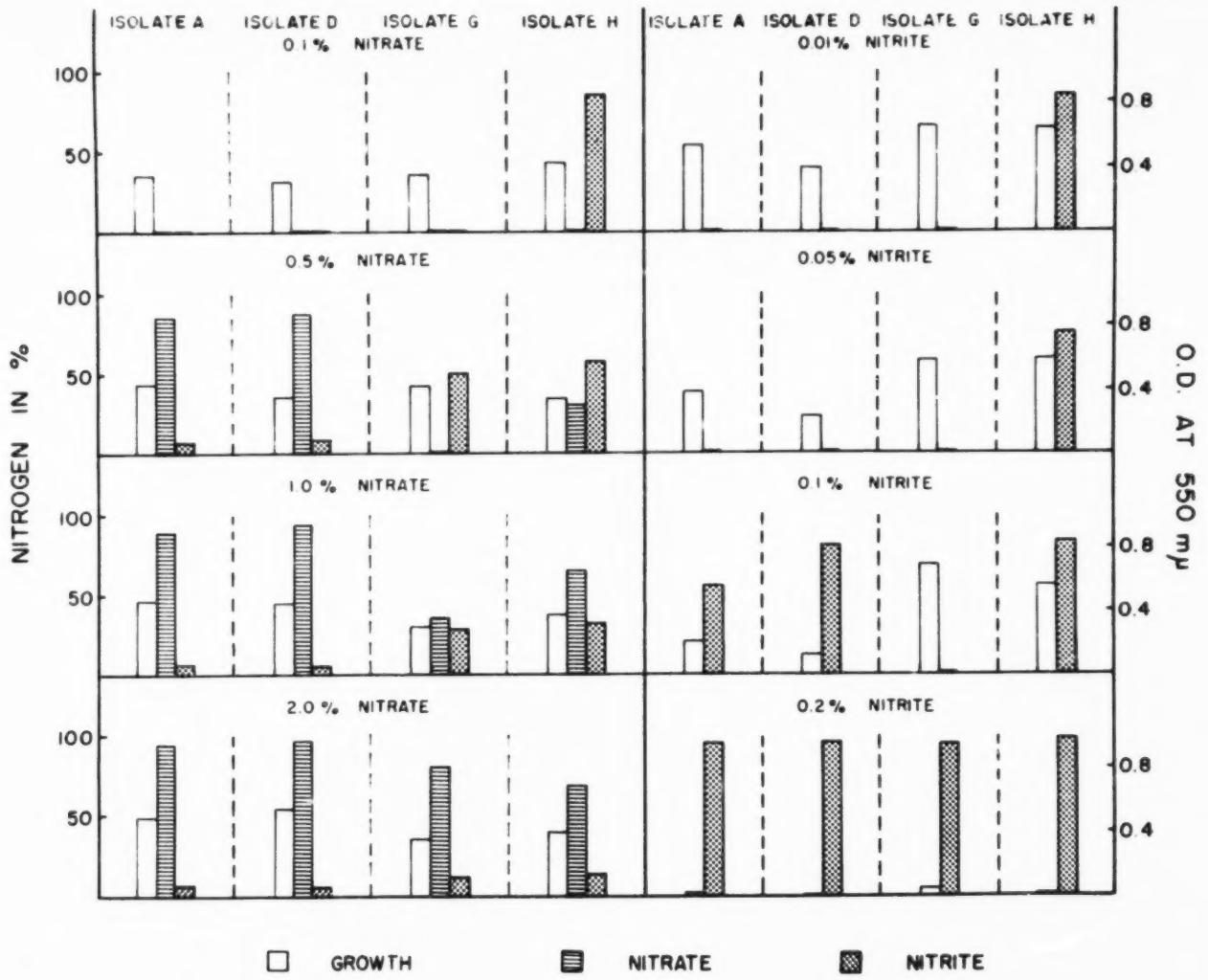

Figure 5: Influence of different nitrate and nitrite concentrations on the growth and denitrifying characteristics of the isolated bacteria (samples taken from 3-day-old anaerobic cultures).

Interactions of Microorganisms During Denitrification

Little attention has been given to the interaction of denitrifying organisms within an ecosystem. Multiple possibilities of combinations between microbes are possible; therefore, these experiments are difficult to perform for clear conclusions on interactions.

The experiments reported here appear to be the first attempt to investigate this problem. Several interesting observations appeared during the combined growth of the isolated microorganisms under anaerobic conditions (Figure 6). Nitrate disappeared under all culture combinations, but the formed nitrite was attacked differently. For example, Isolate G produced a nitrite-nitrogen concentration of 55 to 75 ppm after 2 days (Figure 6) while very little was produced by this organism in combination with either Isolates A or D. This indicates that Isolates A and D are able to degrade nitrite as soon as it is formed. Nitrite concentration increased temporarily to 95 ppm in a mixed culture of Isolates G and H.

As previously described, Isolate H produced nitrite which accumulated in the medium. A mixture of Isolates D and G was able to degrade the formed nitrite. However, when Isolate A was cultured in combination with Isolate H, the nitrite concentration was not reduced. This indicates that one microbe may inhibit a second denitrifier. The formation of nitrite by one bacterium may be toxic for another. Consequently, the possible interactions of microbes have to be carefully considered if nitrate volatilization of a mixed population is to be evaluated.

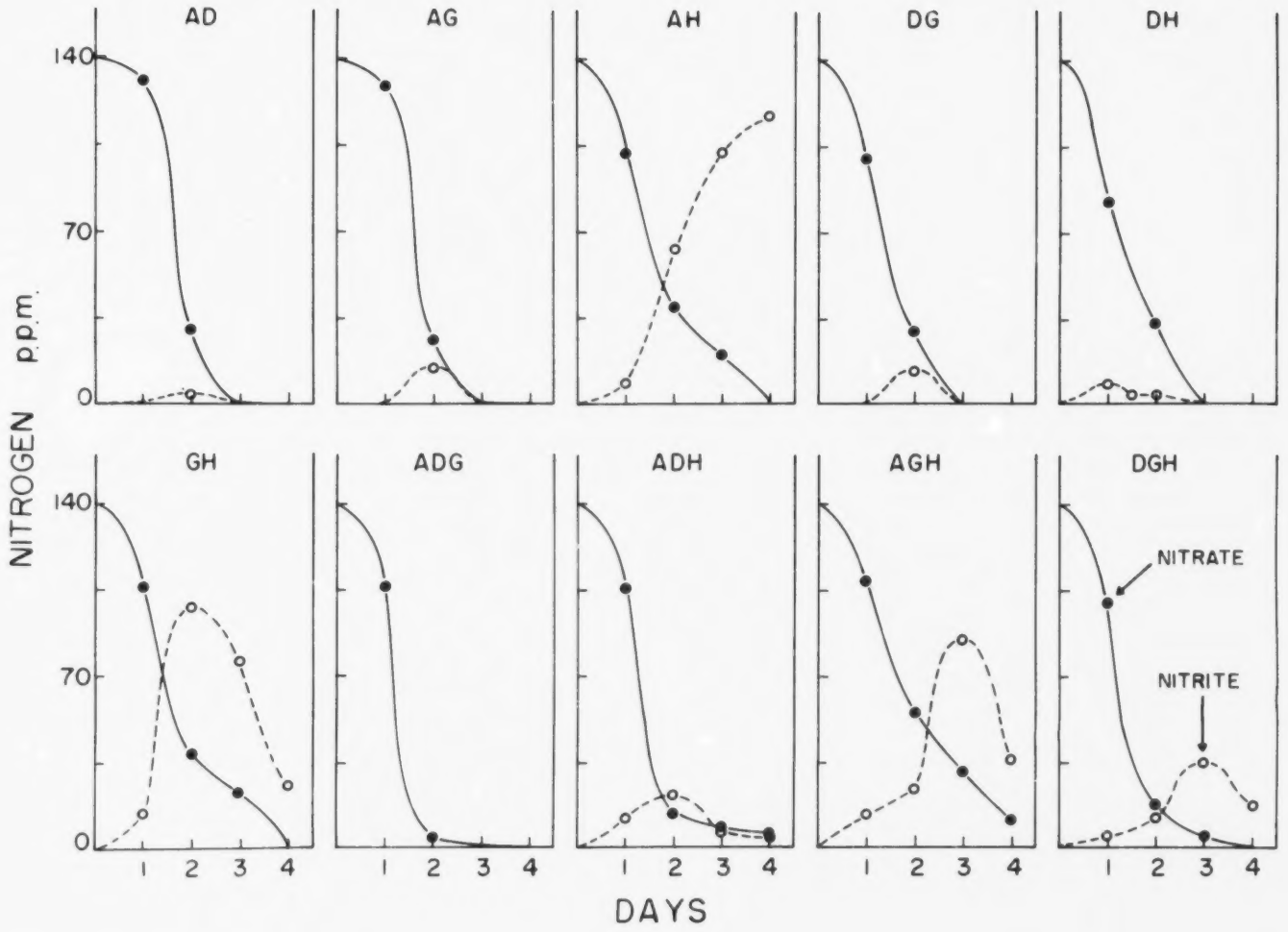

Figure 6: Denitrifying characteristics during combined growth of the investigated bacteria (the letters indicate the combination of the tested isolates).

SECTION VIII

NITROUS OXIDE RELEASE BY SOIL FUNGI

The following study was made to investigate the possible participation of fungi in nitrogen volatilization or denitrification processes in soil.

Although certain bacteria reduce nitrate and nitrite to nitrogenous gases by the process of denitrification, there is still no definite evidence that fungi can perform a similar reaction (Nicholas, 1965). A dissimilatory nitrate reductase can be present if fungi are grown submerged where oxygen supply is limited (Walker and Nicholas, 1961; Nicholas and Wilson, 1964), but the reduction process halts in this case with the formation of nitrite. A nitrite reductase was also isolated from Neurospora (Nason et al., 1954), but nitrite was reduced in this case to ammonia which indicates an assimilatory pathway. Most fungi can utilize nitrate and nitrite as a nitrogen source under aerobic conditions in an assimilatory process, but little is known about the anaerobic growth of fungi and the possible formation of nitrogenous gases.

Experimental Procedure

Two Fusarium species, F. oxysporum and F. solani, were isolated from soil samples collected from 15 to 50 cm below the surface of a silty loam soil. The isolation was performed at 30°C under anaerobic conditions using Brewer anaerobic Petri dishes with Czapek-Dox agar containing 0.2% (w/v) sodium thioglycollate. To suppress the growth of bacteria, 0.1% (w/v) streptomycin (Pfizer) and 100 units ml^{-1} of penicillin (Pfizer) were added to the growth medium. Subsequently, it was possible to transfer and culture the fungi under the described anaerobic conditions. The liquid growth media were inoculated with spore suspension from agar-grown slants.

For the growth experiments two different media were used: Medium A; a modified medium of Cove (1966) containing dextrose, 10 g; peptone (Difco), 2 g; yeast extract (Difco), 3 g; casein hydrolysate, 1.5 g; KCl, 0.52 g; $MgSO_4 \cdot 7H_2O$, 0.52 g; KH_2PO_4, 13.2 g; and 1 ml trace elements solution ($Na_2B_4O_7 \cdot 10H_2O$, 400 mg; $CuSO_4 \cdot 5H_2O$, 500 mg; $FeSO_4 \cdot 2H_2O$, 400 mg; $MnSO_4 \cdot 2H_2O$, 800 mg; $ZnSO_4 \cdot 7H_2O$, 9 g; $Na_2MoO_4 \cdot 2H_2O$, 800 mg/l. distilled water) in 1 liter distilled water; Medium B contained nutrient broth (Difco) enriched with 1% (w/v) dextrose, 0.3% (w/v) yeast extract and 0.1% (v/v) of the same trace elements solution used in Medium A.

Potassium nitrate was added as required before autoclaving, but sodium nitrite was added through millipore filteres (0.22 μ pore size, Millipore Corp., Bedford, Mass.) after steam sterilization of the medium.

For aerobic growth, the inoculated media were incubated at 30°C on a rotary incubation shaker (200-250 osc min^{-1}). 'French Square' flasks (120 ml) containing 50 ml medium were used for incubation under anaerobic conditions. The flasks were closed with a two-hole rubber stopper in which cylindrical septum plugs for gas chromatographs were tightly inserted for gas flushing and sampling. Inside the flask a glass tube was fixed in the inlet plug hole which extended into the medium. To flush with helium, a syringe needle was inserted into the inlet plug and excess gas and air were released from the flask through the outlet plug which was also temporarily provided with a syringe needle. After flushing for 2 min, both needles were immediately removed. Sampling with a gas-tight syringe for gas-chromatographic analysis indicated that all air was removed by this method.

For some experiments, the flasks were not flushed but tightly closed; in this case it was assumed that the available amount of oxygen would provide a better beginning growth for the mycelium, and after use of

oxygen a nearly anaerobic environment was generated. These conditions were characterized as 'initially aerobic'.

All flasks were incubated at 30°C. Carbon dioxide was absorbed in small test tubes containing 1.5 ml 3 N KOH which were placed into the incubation flask.

For experiments with resting cells, fungi were cultured in 2-liter Erlenmeyer flasks under 'initially aerobic' conditions for 5-7 days. The mycelium was harvested by filtration and washed three times with 0.02 M phosphate buffer, pH 7.0. A 10-ml Erlenmeyer flask with a rubber stopper containing a septum for flushing and gas sampling was used as the reaction vessel. Nitrite-transforming activity was assayed at 30°C by incubating 1 g of mycelium in 0.02 M phosphate buffer (pH 7.0) with the equivalent of 100 parts/10^6 N of the N-containing substrate in a final volume of 4 ml. Boiled mycelium, a mycelium without substrate, and substrate alone were included as controls in all assays. At least two replicates were included in each treatment and experiments were repeated two or three times. Anaerobic conditions were also provided by flushing with helium.

Experiments with Growing Cultures

The growth of Fusarium oxysporum and F. solani was observed under aerobic and anaerobic conditions in different liquid media which had been enriched with 0.1% (w/v) KNO_3 or $NaNO_2$. The fungi grew well in the various media, but oxygen supply affected their appearance. If the fungi were cultured in a well aerated atmosphere on a rotary shaker (250 osc min^{-1}), the mycelium grew fast, and a thick paste-like mixture of mycelium and medium was obtained within 3 days. If the fungi grew under conditions of limited oxygen supply but with initially aerobic conditions, the yield of cells was approximately ten times less after 6 days than under aerobiosis. Flushing of the incubation flask with

helium for 5 min provided conditions which were considered anaerobic, and in this case even less growth was observed.

When nitrite was supplied to F. oxysporum grown at 30°C in either aerobic or initially aerobic conditions, nitrite disappeared from the culture solution within 6 days. In the case of F. solani grown under similar conditions, nitrite disappeared from the aerobic cultures, while a small amount of nitrite remained in the initially aerobic cultures (Table 5). There was no decrease of nitrate in the fungal growth medium, however, even though both isolates grew well in the presence or absence of oxygen. The lack of nitrate disappearance indicates that the investigated fungi do not have a nitrate-reducing enzyme system.

The growth of the fungi in relation to gas production under initially aerobic conditions was studied in more detail (Table 6). After 3 days of growth a small amount of N_2O production increased continuously thereafter. It is possible to deduce from the dry weight determinations of the harvested mycelium that the growth of the fungi stopped after 7 days, but the amount of N_2O increased further. After 10 days of incubation approximately 12.8 percent of the nitrite-nitrogen supplied could be detected as N_2O and all nitrate had disappeared. No attempt was made to determine the amounts of nitrogen present in the medium or assimilated by the fungi, since we were interested in the possible volatilization of the nitrite-nitrogen. The presence of molecular nitrogen could readily be detected by gas chromatography. However, conclusions were difficult to make because the 'initially aerobic' conditions were such that N_2 was present at the beginning of the experiment, but an increase in molecular N_2 was still indicated. Under anaerobic conditions N_2O release was much less—which seems to be related to less growth—and the formation of N_2 was not significant. Attempts were made to trap nitric oxide or nitrogen dioxide in alkaline permanganate solution, but our results were negative.

TABLE 5: EFFECT OF GROWTH OF FUSARIUM ISOLATES UNDER AEROBIC AND INITIALLY AEROBIC CONDITIONS FOR 6 DAYS AT 30°C ON THE DISAPPEARANCE OF NITRATE AND NITRITE FROM THE GROWTH MEDIUM.

	NO_3'-N in parts/10^6		NO_2'-N in parts/10^6	
	Initially Aerobic	Aerobic	Initially Aerobic	Aerobic
F. oxysporum	110	109	0	0
F. solani	107	108	26	0
Uninoculated Control	106	119	224	226

TABLE 6: NITRITE DISAPPEARANCE AND NITROUS OXIDE FORMATION DURING GROWTH OF FUSARIUM OXYSPORUM UNDER INITIALLY AEROBIC AND ANAEROBIC CONDITIONS.

Conditions of incubation	Period of incubation (days)	Dry weight of mycelium (mg/ml)	Oxygen concentration (%)	Nitrite concentration (µg N/flask)	N_2O* formed (µg N/flask)	Per cent of nitrite-N transformed to N_2O
Initially aerobic	0	0.0	20.0	7500	0	0.0
	3	7.6	12.0	7000	29	0.4
	5	21.8	3.5	3500	515	6.9
	7	37.4	1.6	125	742	9.9
	10	37.3	0.08	0	956	12.8
Uninoculated control	10	0.0	20.0	7520	0	0.0
Anaerobic	0	0.0	0.0	5030	0	0.0
	3	0.0	0.0	4150	7	0.9
	6	0.0	0.0	3440	128	1.6
	9	23.9	0.0	1300	280	3.6
	13	28.1	0.0	1130	513	6.5
Uninoculated control	13	0.0	0.0	5000	0	0.0

*No corrections were made for the solubility of N_2O in the medium.

Resting Cell Experiments

When resting cells of F. oxysporum were incubated anaerobically at 30°C in the presence of nitrite, results similar to those of the growth experiments were obtained (Table 7). An increase of nitrous oxide was accompanied by a proportionate disappearance of nitrate. A greater mass of mycelium provoked a stronger reaction: 49.7 mg (dry weight) mycelium produced 35.3 µg N_2O-nitrogen from 400 µg nitrite-nitrogen in 5 hours, while it took 20 hours for 22.7 mg (dry weight) mycelium to form 36.5 µg of N_2O-nitrogen.

If nitrite was substituted in the reaction by other nitrogen-containing substrates like KNO_3, $(NH_4)_2SO_4$ or $NH_2OH \cdot HCl$, at concentrations of 100 parts/10^6N, the formation of N_2O could not be observed after incubation of resting cells of F. oxysporum for 5 hours under similar conditions.

If the fungal mycelium was grown in an initially aerobic medium without nitrite, no conversion of nitrite to N_2O could be detected in a subsequent resting-cell experiment. It was also evident that cells of F. oxysporum cultivated in a medium containing nitrite under well aerated conditions were not able to produce nearly the same amount of N_2O in a replacement-culture experiment as cells grown in the presence of nitrite under initially aerobic or anaerobic conditions.

The production of nitrogenous gases by the reduction of nitrate or nitrite during anaerobic respiration by fungi has not been reported before. The fact that fungi cannot grow as well as some bacteria do under anaerobic conditions (Cochrane, 1958) reduces the possibility that fungi play a major role in denitrification within soil. Nevertheless, participation of fungi in nitrogen volatilization—including possible chemical interactions—cannot be excluded.

TABLE 7: NITRITE REDUCTION AND NITROUS OXIDE PRODUCTION BY RESTING CELLS OF F. OXYSPORUM.

	Time of incubation (hours)					
	0		2 1/2		5	
	µg N/flask	%	µg N/flask	%	µg N/flask	%
Nitrite-nitrogen remaining	400	100	305	76.2	0.0	0
Cumulative N_2O-formation	0	0	21.8	5.5	35.3	8.8

There are several recent reports which described the anaerobic growth of certain fungal species: Mucor rouxii (Bartnicki-Garcia and Nickerson, 1961); Fusarium oxysporum (Gunner and Alexander, 1965); Neurospora crassa (Nicholas and Wilson, 1965); and Aspergillus nidulans (Cove, 1966). The two Fusarium species, F. solani and F. oxysporum, which we isolated from sub-soil, were also capable of growing anaerobically. The fact that it was possible to demonstrate that N_2O was formed during the growth of the fungi as well as by resting cells and that significantly more N_2O was formed under conditions of limited oxygen supply provided an indication that a nitrite-reducing dissimilatory reaction may be involved. Since no N_2O was formed in a resting cell experiment when nitrate, ammonium or hydroxylamine served as substrates, these substances seem to be excluded as intermediates or compounds related to the observed activity.

Denitrifying bacteria are the only microorganisms which have b reported to produce significant amounts of N_2O. The possibility of N_2O production by other organisms or by other physiological reactions has rarely been reported. Nitrous oxide was observed during the oxidation of NH_2OH to nitrite by cell-free extracts of Nitrosomonas europaea (Nicholas and Jones, 1960; Falcone et al., 1963; Anderson, 1964). A chemical reaction between hydroxylamine and nitrite can cause the formation of N_2O, and we suggest that this reaction may explain the results described in previous reports ($NH_2OH + NO_2' \rightarrow N_2O + H_2O + OH'$). In the experiments of Renner and Becker (1970) where nitrite and hydroxylamine were supplied simultaneously, the above chemical reaction was no doubt initiated and the addition of resting cells of Corynebacterium nephridii probably did not initiate N_2O formation in the way they concluded. Furthermore, Nicholas (1965) reported that N_2O is also likely to be formed nonenzymatically when some intermediate at the nitroxyl or hyponitrite level accumulates.

No fungi have been reported to denitrify, and the observed formation of N_2O in this study does not imply such a mechanism. The possible

release of N_2O by a chemical side-reaction—as mentioned above—cannot be excluded but requires experimental proof. The finding, however, that more N_2O is produced when the fungal cells were cultured under initially aerobic or anaerobic conditions could indicate that a certain degree of nitrite respiration may be involved, but it is not possible for us to make any firm conclusions.

Nevertheless, it was possible to demonstrate that fungi can participate—at least indirectly—in nitrite volatilization, and this factor may be of importance if one is interested in influencing the nitrogen content of the soil.

SECTION IX

DENITRIFICATION IN SOIL SAMPLES

The volatilization of nitrogen-containing compounds from soil into nitrogenous gases is attributed to biological as well as chemical reactions. Denitrification, chemical nitrite decomposition, and volatilization of ammonia seem to be the predominant processes, but there is no clear knowledge of which one of the volatilization mechanisms is of a greater practical importance. Some investigators hold mostly the biological reaction of denitrification responsible for nitrogen losses from the soil (Nommik, 1956; Allison, 1966), whereas other studies tend to emphasize more the chemical volatilization (Nelson and Bremner, 1969 and 1970; Bulla, Gilmour and Bollen, 1970). There is little doubt that both processes are influenced by factors like pH, organic matter and others, but only a few and incomplete approaches have been made in order to establish to what extent these factors are of dominating influence for the biological or chemical reaction.

It was the purpose of this investigation to clarify if nitrite, which is an intermediate during nitrogen transformation in soil, is volatilized rather by biological or chemical means, by comparing its disappearance in sterile and non-sterile soil systems under various conditions.

Experimental Procedure

The soil used in all the experiments had an original pH of 7.0, an organic-carbon content of 1.60%, and with a texture composed of 7.4% sand, 67.5% silt and 25.1% clay. Before use, the soil was air-dried for 48 hours in layers of 1 cm thickness and passed through a 2-mm sieve. The original air-dried soil contained 50 µg of NO_3-N per gram.

One portion of the air-dried soil was adjusted to pH 5.0 by titration with approximately 40 ml of 0.2 N HCl per 100 g of soil. After

adjustment of the pH, the chloride was removed by leaching and subsequently the soil was oven-dried at 45°C. A recheck of the pH after drying gave a value of 5.0. In order to obtain soil with a pH of 8.3, increments of powdered $Ca(OH)_2$ were added to a 1:2 soil-to-water mixture until the desired pH was obtained.

For some experiments a portion of the soil in the neutral and alkaline pH-category was leached with distilled water in order to decrease the content of water-extractable nitrogen, particularly the nitrate which was present. Since all of the acidified soil had been leached, one portion received 1 mg of nitrate dissolved in 10 ml H_2O per 20 g soil to restore its nitrate level to that in the original soil. The same 10 ml of H_2O also contained 5 mg of nitrite-nitrogen. Where only nitrite was added, 5 mg nitrite dissolved in 10 ml H_2O was added to each 20 gram portion of soil.

Sterilization of soil was achieved by autoclaving the samples at 121°C and 15 lb steam pressure three times for 30 minutes with intervals of at least eight hours within a two-day period. Nitrite and nitrate solutions which were added to autoclaved soil were sterilized by millipore filtration (0.2-μ pore size, Millipore Corp., Bedford, Mass.).

Soil samples which were incubated under anaerobic conditions at 30°C in 125 ml flasks were sealed by a rubber stopper in which a septum was inserted for gas sampling. Anaerobic conditions were prepared by flushing helium gas through the flasks until all air was exchanged by helium as determined by gas sampling and subsequent gas-chromatographic analysis. In all flasks a constant positive pressure of helium gas was applied before incubation (1.25 atm) and the resulting change of gas pressure by chemical or biological activity did not appear to change the applied gas pressure significantly. The gases used for the calibration curves were also kept under the same conditions. The volume of the gas phase was measured in each flask and the amount of gas formed was calculated from the representative analyzed sample. If incubation

was performed under aerobic conditions, the samples were put into 125 ml flasks closed with foam tube plugs and kept in an incubation chamber which was continuously flushed with filtered air; in order to diminish the drying of the soil, a large pan of water was placed in the chamber to increase the humidity. Two replicates were included in each treatment and the same experiment was repeated once; the results reported represent average values.

The determination of nitrite and nitrate in soil samples was performed by extraction of 20 g of air-dried soil with 40 ml distilled water during 30 min on a 'Wrist-Action' shaker; subsequently the soil was separated from the aqueous phase by cenitrifugation and the supernatant analyzed.

Biological versus Chemical Nitrite Decomposition

Since it was the major purpose of this investigation to distinguish between biological and chemical nitrite volatilization, all experiments described in this section—except one—were carried out with sterile and non-sterile soil. It is evident from Table 8 that under anaerobic conditions at a neutral pH there was very little nitrogen-degrading activity in the autoclaved soil while all nitrate and nitrite disappeared in the non-sterile soil. It is also interesting to note that during the first 6 days the dominating gas produced in the latter soil was nitrous oxide, which changed almost entirely to molecular nitrogen during further incubation.

One of the most important environmental factors influencing biological as well as chemical nitrite volatilization is the pH of the soil. Therefore, the pH of the soil was adjusted from that of the natural soil, pH 7.0., to values of 5.0 and 8.3, and the soil provided with nitrite. The decrease of nitrite and the formation of nitrogenous gases were observed during anaerobic incubation (Figure 7). There is no apparent difference between sterile and non-sterile soil under

TABLE 8: PRODUCTION OF NITROGENOUS GASES FROM AUTOCLAVED AND NON-AUTOCLAVED SOIL AT pH 7 UNDER ANAEROBIC CONDITIONS.

	Remaining amount of nitrite and nitrate				Cumulative gas production in µg N/g soil							
	0 days		12 days		3 days		6 days		9 days		12 days	
Soil*	NO_2^--N µg/g soil	NO_3^--N µg/g soil	NO_2^--N µg/g soil	NO_3^--N µg/g soil	N_2O	N_2	N_2O	N_2	N_2O	N_2	N_2O	N_2
Autoclaved	250	50	225	50	0	0	0	10	0	18	0	20
Non-autoclaved	250	50	0	0	84	22	130	79	78	219	59	250

*Each soil initially contained 50 µg NO_3^--N/g and 250 µg NO_2^--N dissolved in 0.5 ml of H_2O was added to each gram.

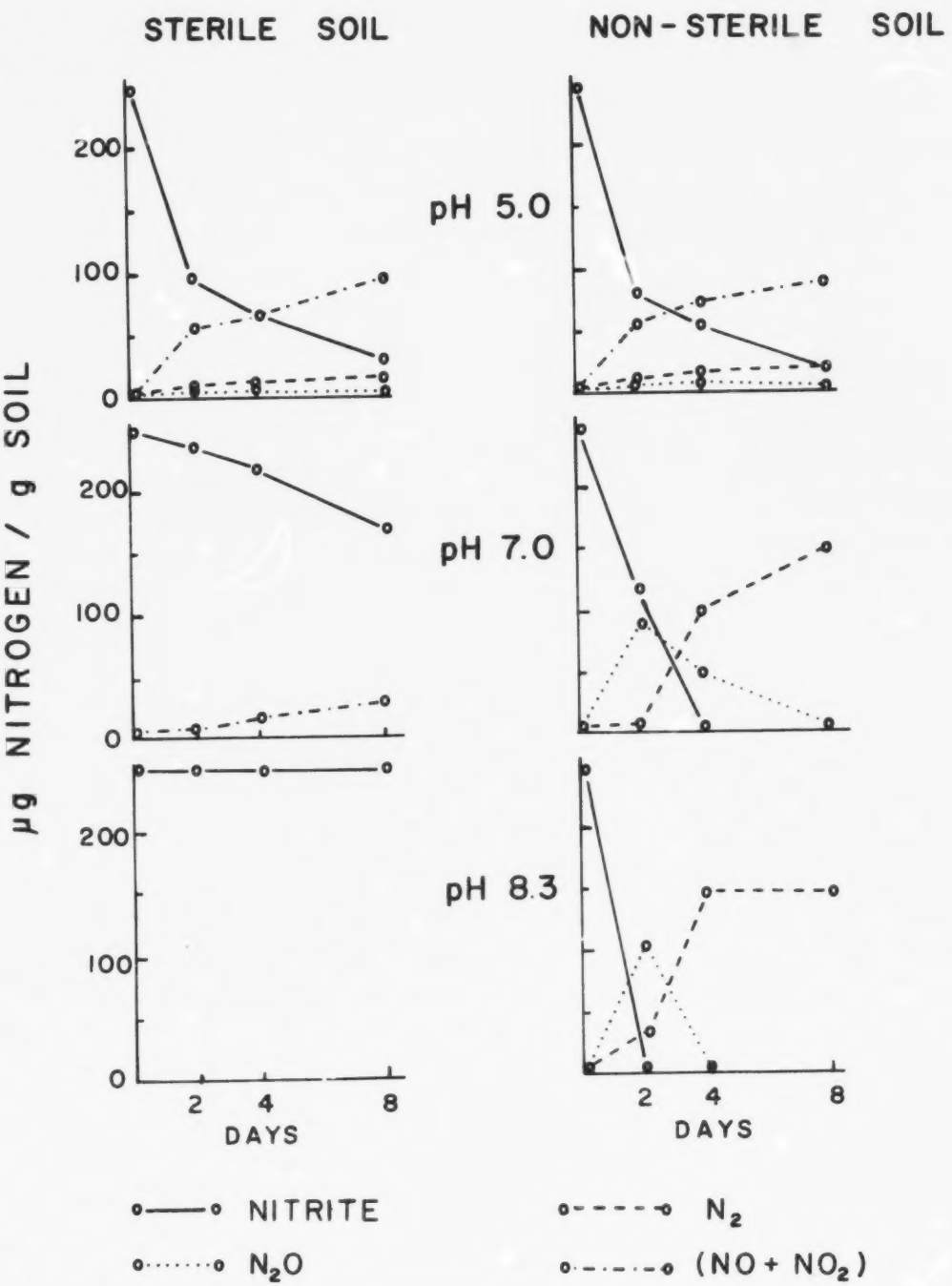

Figure 7: Formation of nitrogenous gases from nitrite during anaerobic incubation of leached sterile and non-sterile soils with different pH values.

acidic conditions (pH 5.0) in the rate of nitrite disappearance, but there are considerable differences in a neutral or alkaline pH. The nitrite concentration decreased rapidly within the first 2 days of incubation at pH 5.0 in sterile and non-sterile soils, but then the rate of decomposition slowed. In an autoclaved soil at pH 7.0 the rate of nitrite disappearance was small and no decrease occurred at a pH 8.3. The soils which were biologically active lost all nitrite within 4 days at a pH 7.0 or within 2 days at a pH 8.3.

The composition of the nitrogenous gases which were evolved during incubation also varied considerably according to the pH. Gas release from sterile soils in a neutral or alkaline environment was small or nonexistent, respectively. In non-sterile conditions the formation of N_2O or N_2 was dominant. Again it was observed that during the initial period of incubation nitrous oxide was the major gas generated, but later N_2 was the predominant volatilization product. These findings indicated that denitrification is very likely responsible for this transformation, since essentially microorganisms are able to reduce nitrous oxide to molecular nitrogen.

No production of NO or NO_2 could be found in non-sterile neutral and alkaline soils, and only a very small amount of these gases was trapped by $KMnO_4$ in a sterile and neutral soil. At a pH 5, however, the major gaseous products evolved in sterile and non-sterile samples were NO and NO_2, and only very small amounts of N_2 and N_2O were released. The reason for this appearance seems to be the suppression of biological activity in an acid environment, and it indicates that nitrite volatilization at a low pH is caused mainly by chemical reactions.

As was mentioned earlier, the original soil contained naturally a small amount of nitrate. To evaluate the possible influence of this nitrate upon nitrite volatilization, an additional experiment was performed. Portions of the pH 7 and 8.3 soils which were leached to provide soils

free of nitrate and a portion of the pH 5 soil which had been leached in adjusting the pH, all received nitrate-nitrogen equivalent to that found in the natural soil. Table 9 shows that the acid soil to which nitrate was not added was more active in chemical nitrite degradation than the soil which received nitrate. In the neutral sterile soil there was relatively little difference between the unleached (nitrate-containing) and leached (nitrate-free) soil with respect to total nitrite disappearance. In neutral, non-sterile soils nitrite disappeared more completely in the leached soil, but there was no difference in the quantity of nitrogenous gases recovered. With the sterile, alkaline soil there was virtually no difference in the behavior of the leached and unleached soil. With the non-sterile, alkaline soil nitrite disappearance was similar in leached and unleached soil, but in the nitrate-containing soil N_2 and N_2O was observed, whereas in the nitrate-free soil only N_2 was recovered after 4 days. This phenomenon indicates that the presence of nitrate influences the composition of nitrogenous gases and that the conversion of nitrite to N_2 is faster in the absence of nitrate.

The formation of small amounts of nitrate in some samples under anaerobic conditions was not further investigated but may have been due to spontaneous decomposition of the nitrite to produce nitrate and nitric oxide.

Influence of Oxygen on Nitrite Volatilization

Another environmental factor which is of major importance in influencing nitrite volatilization concerns the oxygen supply in soil. The decrease of nitrite was followed in aerobic and anaerobic conditions with sterile and non-sterile soil of different pH values (Table 10). Only soil samples at a neutral or alkaline level which were not autoclaved respond to changing oxygen conditions. No disappearance at all of nitrite was found in aerobic, alkaline conditions and only 10% nitrite disappearance was observed at a pH of 7.0 in neutral,

TABLE 9: NITRATE AND NITRITE TRANSFORMATION AND FORMATION OF NITROGENOUS GASES IN STERILE AND NON-STERILE SOIL, AT DIFFERENT pH VALUES, IN LEACHED AND UNLEACHED SAMPLES INCUBATED UNDER ANAEROBIC CONDITIONS.

		Nitrate-nitrogen in µg/g soil		Nitrite-nitrogen in µg/g soil		Recovery of nitrogenous gases in µg N/g soil after 4 days			
		0 days	4 days	0 days	4 days	as N_2O	as N_2	as (NO_2 + NO)	TOTAL
pH 5	sterile - leached + NO_3^-	50	50	250	140	5	0	22	27
	- leached	0	0	250	90	5	12	94	111
	non-sterile - leached + NO_3^-	50	60	250	130	5	0	10	15
	- leached	0	15	250	70	5	18	77	100
pH 7	sterile - unleached	50	56	250	235	0	0	0	0
	- leached	0	0	250	230	0	0	9	9
	non-sterile - unleached	50	0	250	115	119	18	0	137
	- leached	0	0	250	0	108	34	0	142
pH 8.3	sterile - unleached	50	50	250	250	0	0	0	0
	- leached	0	0	250	250	0	0	0	0
	non-sterile - unleached	50	0	250	20	85	61	0	146
	- leached	0	0	250	0	0	155	0	155

TABLE 10: NITRITE DISAPPEARANCE UNDER AEROBIC AND ANAEROBIC CONDITIONS FROM STERILE AND NON-STERILE SOILS WITH DIFFERENT pH VALUES.

	Disappearance of nitrite-nitrogen after 4 days			
	AEROBIOSIS		ANAEROBIOSIS	
SOILS*	µg/g soil	%	µg/g soil	%
pH 5				
STERILE	165.0	66	180.0	72
NON-STERILE	200.0	80	195.0	78
pH 7				
STERILE	10.0	4	25.0	10
NON-STERILE	25.0	10	250.0	100
pH 8.3				
STERILE	0.0	0	0.0	0
NON-STERILE	0.0	0	250.0	100

*Each soil initially contained 50 µg NO_3^--N and 250 µg NO_2^--N was added per gram.

non-sterile aerobic soil. Under anaerobic conditions very little nitrite disappearance occurred in sterile, neutral or alkaline soil; however, in the same non-sterile soils, nitrite disappeared entirely which undoubtedly was due to denitrification.

Chemical nitrite disappearance under sterile conditions should not be affected by oxygen supply, but at pH 5.0 and pH 7.0, a small influence was detected. A large decrease of nitrite occurred at pH 5.0 under aerobic and anaerobic conditions in both sterile and non-sterile media. This represents a further clear proof that at a low pH chemical nitrite decomposition can be an important factor responsible for nitrite disappearance when substrate conditions are adverse for microbial activity. However, it is worthwhile to point out that biological nitrite disappearance is more effective than chemical degradation when it occurs under optimal conditions, since during the observation period all nitrite disappeared when biological activity was favored in the neutral and alkaline soils. Only approximately 80% of nitrite removal was found in sterile, acid soil where only chemical processes were operating.

Effect of Inoculation of Sterilized Soil on Nitrite Disappearance

In order to clarify further the participation of denitrifying microbes in nitrite volatilization, the investigated soil with different pH values was inoculated with two bacteria, Pseudomonas aeruginosa and a newly isolated denitrifier (Isolate A), whose denitrifying characteristics in culture solution were described previously (Bollag et al., 1970). Both microorganisms were grown in nitrate broth and when nitrate had disappeared from the medium, 1 ml of the bacterial culture was applied to the sterilized soil samples. In additional experiments with the culture solutions it was established that Ps. aeruginosa produced mainly molecular nitrogen, whereas Isolate A generated nitrous oxide. The same physiological activity was observed after inoculation of the soil with the two bacteria (Table 11). Ps. aeruginosa produced

TABLE 11: EFFECT OF INOCULATION OF STERILIZED SOIL AT DIFFERENT pH VALUES WITH DENITRIFYING BACTERIA ON NITRITE DISAPPEARANCE AND NITROGENOUS GAS EVOLUTION AFTER 4 DAYS UNDER ANAEROBIC CONDITIONS.

Soil* and Denitrifiers	Disappearance of nitrite-nitrogen		Recovery of nitrogenous gases in µg N/g soil			
	in µg/g soil	in %	as N_2O	as N_2	as (NO_2^- + NO)	TOTAL
Soil pH 5.0						
Isolate A	125.0	50	5.0	12.0	28.0	45.0
Ps. aeruginosa	125.0	50	5.0	12.0	25.0	42.0
Soil pH 7.0						
Isolate A	10.0	4	5.0	0.0	0.0	5.0
Ps. aeruginosa	250.0	100	0.0	133.0	0.0	133.0
Soil pH 8.3						
Isolate A	250.0	100	169.0	9.0	0.0	178.0
Ps. aeruginosa	250.0	100	0.0	103.0	0.0	103.0

*Each soil initially contained 50 µg NO_3^--N and 250 µg NO_2^--N was added per gram.

most molecular N_2 in a neutral soil, but Isolate A did not reduce nitrite at pH 7. The predominant gas in an acid environment was again nitric oxide or nitrogen dioxide indicating chemical nitrite volatilization, and only minute amounts of N_2O and N_2 were found.

SECTION X

INHIBITION OF METHANE FORMATION IN SOIL BY VARIOUS NITROGEN-CONTAINING COMPOUNDS

The formation of CH_4 in soil under anaerobic conditions and in the presence of organic matter is a very common and well-known process, especially in poorly drained or waterlogged soil. Anaerobiosis appears to be a major condition for the activity of CH_4-producing bacteria, but other factors like redox potential, pH, temperature, moisture, and available organic materials have a major influence on the rate of CH_4 production.

It has been observed that nitrate has to disappear before CH_4 formation begins. This observation was first made by Barker (1941) in studies of cultures of the then called Methanobacterium omelianskii in which very low concentrations of nitrate were used. The suppression of methane production by nitrate in soil was observed by Takai et al. (1956) and Yamane (1957) and more recently by Laskowski and Moraghan (1956) and Bell (1969). The various investigators also concluded that the redox potential has to be reduced considerably before methane production can start.

During an investigation of the denitrifying process we observed inhibition of methane evolution by various nitrogen-containing compounds. This paper describes these findings and correlates the results with the oxidation state of the various nitrogenous substances. The influence of different organic substances on methane accumulation was also investigated.

Soil containing 75 parts/10^6 of nitrate-nitrogen and an added amount of 250 parts/10^6 was incubated under anaerobic conditions with glucose (2.5 mg/g soil) and denitrification was immediately observed as indicated by the evolution of N_2 as well as N_2O and nitrite as

intermediates (Figure 8). When no further denitrification could be observed and the N_2 concentration remained constant, the formation of CH_4 began (Figure 8).

If the initial nitrate concentration was less than 325 parts/10^6 in the soil, denitrification ceased after a shorter time of incubation and the evolution of CH_4 started earlier. Since interest was centered on the formation of CH_4, the soil samples were usually pre-incubated until CH_4 evolution started and then the soils were treated for the different experiments.

Influence of Nitrogen-containing Compounds on Methane Formation

As mentioned previously, CH_4 evolution began only when all nitrate had disappeared from the soil. Subsequently, the activity of CH_4-producing bacteria could be immediately interrupted by the addition of nitrate to the soil. Evolution of CH_4 would not recommence until denitrification was completed.

Table 12 shows the results of an experiment in which the influence of various nitrogen-containing compounds on the formation of methane was investigated. The compounds were added to the preincubated soil. Nitrate, nitrite and the gases nitrous oxide and nitric oxide inhibited the production of methane, but ammonium sulfate and hydroxylamine were ineffective. The oxidation-reduction state of the nitrogenous compounds affected the formation of CH_4 and the concentrations of these substances were also an important factor. Nitrous oxide at concentrations of 50, 100, and 500 parts/10^6 inhibited CH_4 production for 1, 4, and 5 days, respectively, and in the presence of 50, 100 and 500 parts/10^6 nitric oxide it took 4, 5, and 8 days until CH_4 started to accumulate.

It is obvious from Table 12 that nitrate was most effective in suppressing the formation of CH_4; the addition of 500 parts/10^6

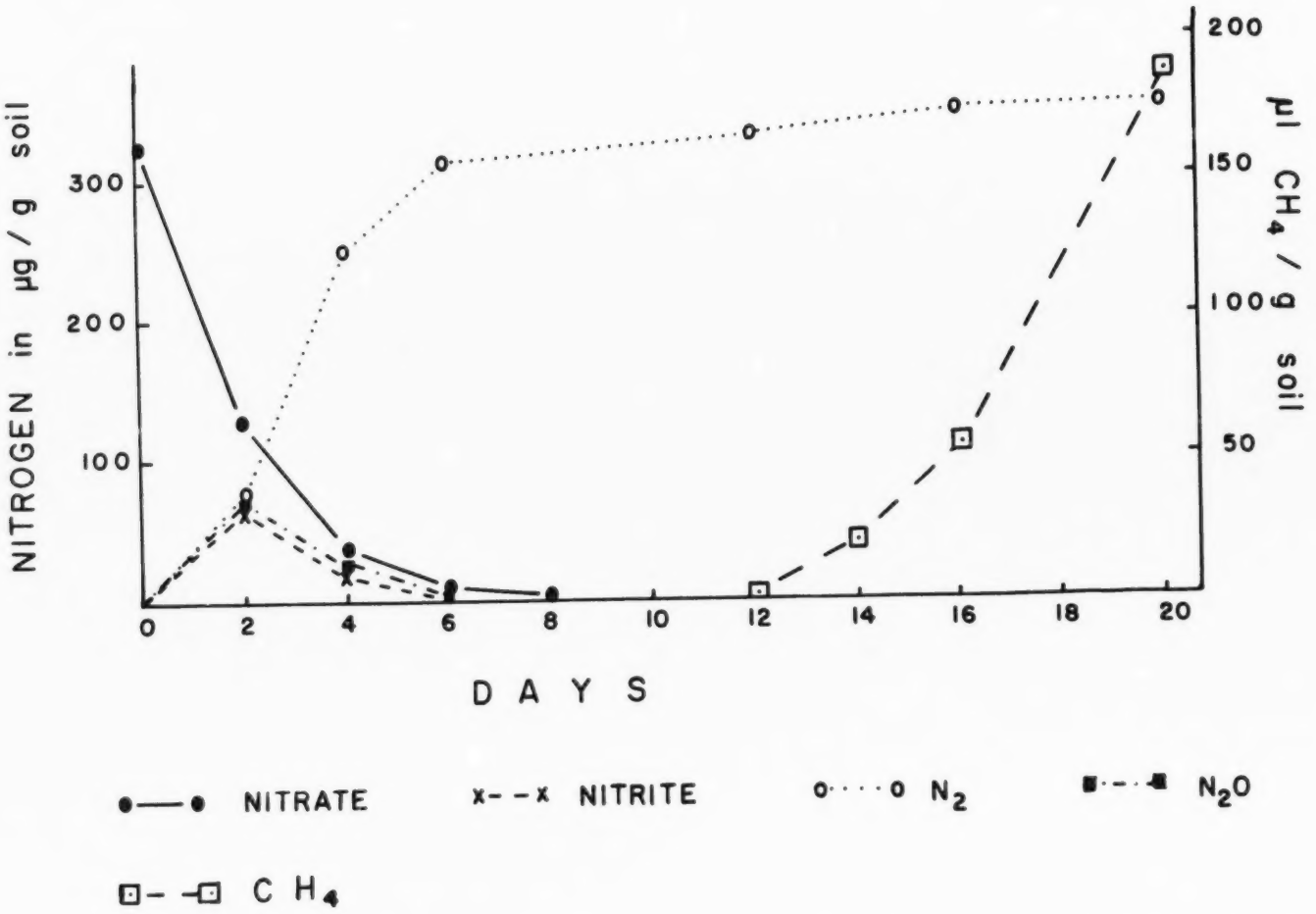

Figure 8: Denitrification and methane formation in soil. (At the start of the experiment the soil contained 325 µg of NO_3'-N/g soil and 2.5 mg glucose/g soil was added.)

TABLE 12: INFLUENCE OF DIFFERENT NITROGEN-CONTAINING COMPOUNDS ON METHANE PRODUCTION IN SOIL.

(μl CH_4 per 1 g of soil)

DAYS OF INCUBATION*	CONTROL	NO_3'-N parts/10^6			NO_2'-N parts/10^6			NO-N parts/10^6			N_2O-N parts/10^6			$(NH_2)OH$ parts/10^6			$(NH_4)_2SO_4$ parts/10^6		
		50	100	500	50	100	500	50	100	500	50	100	500	50	100	500	50	100	500
1	4	0	0	0	0	0	0	0	0	0	0	0	0	4	5	3	5	8	5
2	7	0	0	0	0	0	0	0	0	0	1.1	0	0	9	7	6	9	14	11
3	14	0	0	0	0	0	0	0	0	0	1.9	0	0	15	10	11	12	19	16
4	20	0	0	0	0	0	0	0	0	0	3.3	0	0	18	11	15	18	23	24
5	38	0	0	0	0	0	0	0.6	0	0	4.9	1.0	0	32	19	33	24	30	30
6	49	0	0	0	5.1	0	0	1.8	0.7	0	15	3.8	1.7	N.D.	N.D.	46	N.D.	N.D.	42
8	75	0	0	0	7.0	0	0	7.4	2.7	0	28	9.7	5.9	51	40	62	61	68	56
10	98	3.3	0.6	0	31	0.8	0	18	6.2	2.8	45	29	12	N.D.	N.D.	78	N.D.	N.D.	73
12	145	4.1	0.8	0	63	2.9	0	69	17	9.1	76	51	47	99	92	102	128	119	97
16	311	6.4	1.9	0	191	9.4	1.8	264	55	37	280	132	94	204	188	230	204	212	170

* The soil was preincubated for 8 days under anaerobic conditions after addition of 2.5 mg of glucose per 1 g of soil.

N.D. Not determined.

completely inhibited CH_4 production under the selected experimental conditions.

Influence of Organic Substances on Methane Formation

Three organic compounds (glucose, starch and alfalfa) at concentrations of 2.5 and 10.0 mg per 1 g soil were added to soil and the formation of methane and CO_2 were determined after 8, 12, 16, and 24 days of incubation (Table 13). Starch and alfalfa produced a stronger CH_4 evolution in relation to increasing concentrations, but glucose reacted differently. 2.5 mg of glucose per g of soil showed a similar stimulating effect on CH_4 formation as a corresponding amount of starch and alfalfa, but 10 mg of glucose, which caused an intensive formation of CO_2, almost completely inhibited CH_4 production. If nitrate was added with the organic compounds to the soil, there was—as expected—a delay in the formation of CH_4, but otherwise the results were similar.

TABLE 13: EFFECT OF GLUCOSE, STARCH, AND ALFALFA ON CH_4 AND CO_2 EVOLUTION FROM SOIL.

DAYS OF INCUBATION	CONTROL		GLUCOSE				STARCH				ALFALFA			
			2.5 mg/g soil		10.0 mg/g soil		2.5 mg/g soil		10.0 mg/g soil		2.5 mg/g soil		10.0 mg/g soil	
	CH_4	CO_2	CH_4	CO_2	CH_4	CO_2	CH_4	CO_2	CH_4	CO_2	CH_4	CO_2	CH_4	CO_2
	µl/g soil													
8	0	232	4.6	457	0.8	1086	0.6	277	10	465	6.1	333	7.1	506
12	0.6	299	11	587	2.2	1147	4.4	386	20	546	13	428	39	554
16	13	301	112	631	5.7	1175	89	416	179	672	123	554	414	729
24	101	354	880	744	4.1	1148	429	518	1084	850	418	520	1351	878

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